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Entoloma shandongense T. Bau & J.R. Wang (*Agaricales*, *Entolomataceae*): A new distributional record from India

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Abstract: This study reports *Entoloma shandongense* (first described from China) as a new record for the macrofungal mycota from India. A phylogenetic analysis based on ITS show that *E. shandongense* can be readily distinguished from other blue coloured morphologically similar species, such as *E. atrum*. A detailed taxonomic description with illustrations and an artificial key to blue-coloured *Entoloma* species from India and surrounding areas are provided.

Key words: new record; nrDNA; phylogenetic analysis; West Bengal

The genus *Entoloma* P. Kumm. s.l. (*Basidiomycota*, *Agaricales*) comprises approximately 1,500 species (Noordeloos and Morozova 2010) and is considered to be worldwide in distribution (Noordeloos 1981). Its cosmopolitan nature is observed through studies in South America (Horak 1982), the Pacific Northwest and Alaska (Largent 1994), the Netherlands (Noordeloos 1980), the Mascarenes and the Seychelles (Noordeloos and Hausknecht 2007), Tasmania (Noordeloos and Gates 2007, 2009), Primorsky Territory of the Russian Far East (Noordeloos and Morozova 2010), Brazil (Putzke and Putzke 2000; Alves and Nascimento 2012), China (Li et al. 2009; He et al. 2012; Wang and Bau 2013), and Sri Lanka (Pegler 1986). However, vast areas such as India, Africa, South America and South East Asia are still under-explored (Noordeloos and Morozova 2010). The diagnostic features of angular spores, basidiospore wall evenly cyanophilic, and pinkish spore-print make the genus easy to recognize. Previous molecular works suggest that the genus is monophyletic (Baroni and Matheny 2011; Co-David et al. 2009). As of this date there are reports of ca. 76 species of *Entoloma* from India (Pegler 1986; Manimohan et al. 1995, 2002, 2006; Raj and Manimohan 2012; Farook et al. 2013; Pradeep et al. 2012, 2013), most of which were described as new to science. During a 10-year-long project (Khatua et al. 2015), the authors collected an interesting blue coloured species of *Entoloma* from the state of West Bengal, eastern India. After thorough consultation of available literature (Pegler 1986; Manimohan et al. 1995, 2002, 2006; Raj and Manimohan 2012; Farook et al. 2013; Pradeep et al. 2012, 2013; Wang and Bau 2013), the species was identified as *Entoloma shandongense* T. Bau & J. R. Wang and further confirmed by molecular (ITS sequence) data. This collection constitutes the second record of the species and expands its geographical distribution to India. We present morphological and molecular studies of the collected taxon, and an artificial key to blue-coloured *Entoloma* species from India and surrounding areas to aid in future identification by others.

The specimen under consideration was collected during August 2014 from Kolkata, West Bengal, India. The material was photographed in the field using a digital camera and extensive notes on the basidiomata were accomplished before drying. Colour terms (mostly) follow the Royal Botanic Gardens Edinburgh colour chart (Henderson et al. 1969). Microscopic features were obtained from reviving dried material in 95% ETOH and then dH_2O and then by mounting free-hand sections of basidiocarp tissues in 5% KOH, Melzer's reagent and Congo Red. Basidiospores size are based on 30 measurements from each of the three collected basidiomata of a single collection and given as a mean value (underlined); values in parentheses indicate minimum or maximum measured values. Q value denotes length/width ratio of the basidiospores. The voucher specimen has been deposited in the Calcutta University Herbarium (CUH).

Genomic DNA was extracted following Dutta et al. (2014). Selective PCR amplification of nrDNA ITS region was performed according to the modified method of Gardes and Bruns (1993) using the primer pairs ITS1F (forward) and ITS4B (reverse). A hot start of 4 min. at 94°C was followed by 30 cycles consisting of 30 sec. at

94°C, 30 sec. at 56°C, 1 min. at 72°C, and a final elongation step of 5 min. at 72°C. PCR products were purified using QIAquick® Gel Extraction Kit (QIAGEN, Germany) and were used for automated DNA sequencing on ABI3730xl DNA Analyzer (Applied Biosystems, USA) using primers identical with amplification for nrDNA ITS region. The generated sequence was edited manually using BioEdit sequence alignment editor version 7.0.9.0 (Tom Hall, Ibis Biosciences, Carlsbad, USA). The edited sequence was then used for BLAST searches in the GenBank database. The newly generated sequence was deposited in GenBank (KP241852). The newly generated ITS sequence (615 bp) and those retrieved from GenBank based on a BLAST search of KP241852 (19 ITS sequences) were chosen for purpose of phylogenetic analysis. The closest hit of KP241852 was *Entoloma shandongense* T. Bau & J.R. Wang (GenBank KC257440; sequence identity = 602/607 (99%); gaps = 4/607(0%)). *Tricholoma vaccinum* (Schaeff.) P. Kumm. and *Lyophyllum decastes* (Fr.) Singer were selected as outgroup taxa for rooting purpose following He et al. (2012). Altogether twenty-three sequences were aligned with ClustalX (Thompson et al. 1997) using default settings, followed by additional manual adjustments. The end of the final alignment was trimmed to create a dataset of 626 bp that included 458 positions.

Maximum Parsimony (MP) analysis was conducted using MEGA6 (Tamura et al. 2013). Searches employed a heuristic search method with all characters weighted equally, gaps treated as missing data, search level 1 where the initial trees were obtained by the random addition of sequences (10 replicates), tree bisection reconnection (TBR), and collapse of zero length branches. The branch lengths were calculated using the average pathway method (Nei and Kumar 2000) and are in the units of the number of changes over the whole sequence.

Besides MP, phylogenetic analyses were also carried out using the maximum likelihood (ML) method to determine whether different methods (MP vs. ML) alter the resulting phylogenetic tree. ML bootstrapping analysis was done based on the general time reversible model (Nei and Kumar 2000) where the initial tree(s) for the heuristic search was obtained by applying the neighbour-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4770)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.0000% sites). In both analysis, all positions containing gaps and missing data were eliminated and a bootstrap test of 1,000 replicates were performed to obtain the percentage of replicate trees for clustering the associated taxa (Felsenstein 1985). Data obtained from both ML and MP analyses (Bootstrap percentages of ≥ 50%) has been indicated in Figure 1 (next to the branches).

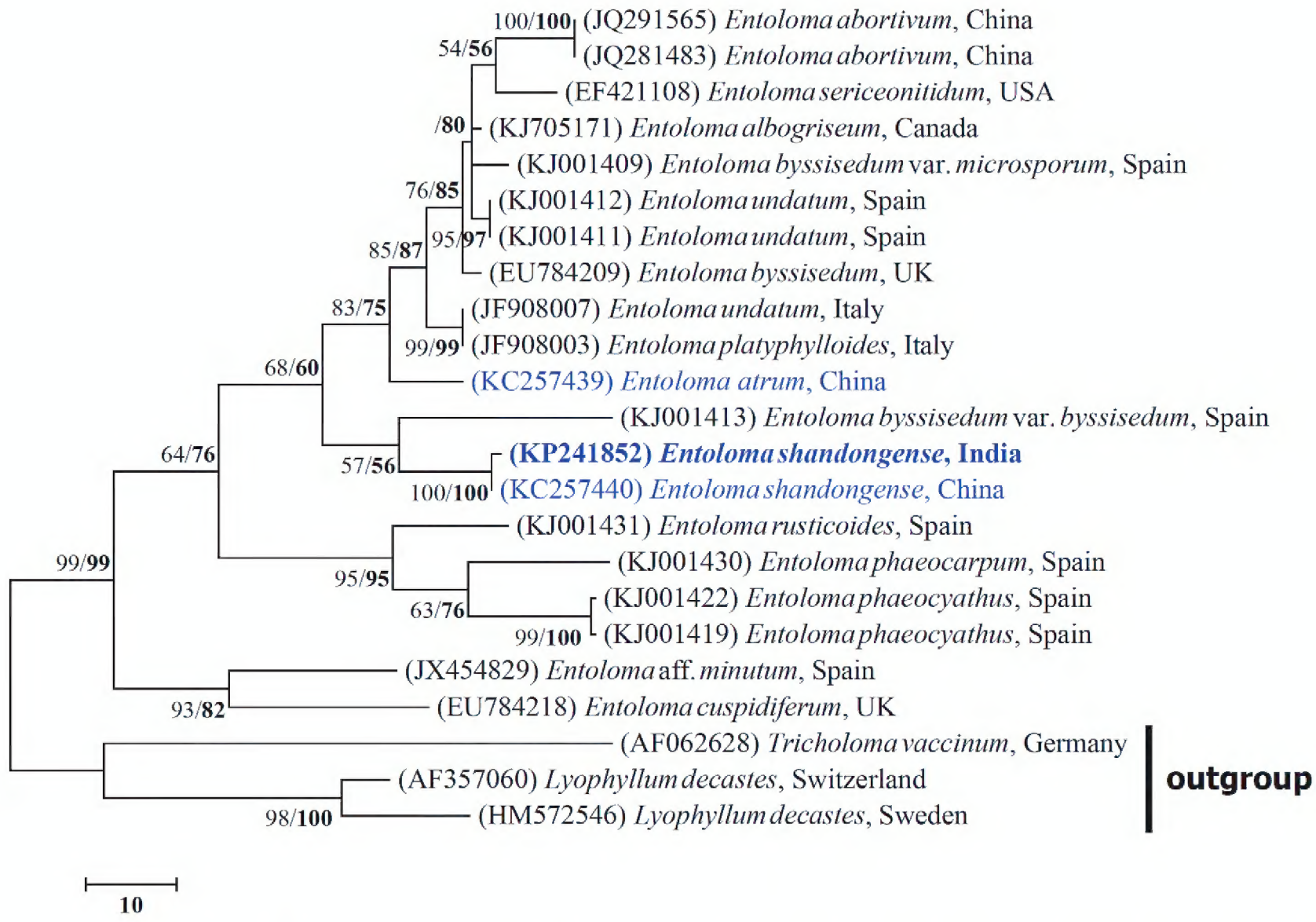

100/100 (JQ291565) Entoloma abortivum, China
54/56 (JQ281483) Entoloma abortivum, China
(EF421108) Entoloma sericeonitidum, USA
/80 (KJ705171) Entoloma albogriseum, Canada
(KJ001409) Entoloma byssisedum var. microsporum, Spain
76/85 (KJ001412) Entoloma undatum, Spain
95/97 (KJ001411) Entoloma undatum, Spain
85/87 (EU784209) Entoloma byssisedum, UK
83/75 (JF908007) Entoloma undatum, Italy
99/99 (JF908003) Entoloma platyphylloides, Italy
68/60 (KC257439) Entoloma atrum, China
(KJ001413) Entoloma byssisedum var. byssisedum, Spain
64/76
57/56 (KP241852) Entoloma shandongense, India
100/100 (KC257440) Entoloma shandongense, China
(KJ001431) Entoloma rusticoides, Spain
99/99
95/95 (KJ001430) Entoloma phaeocarpum, Spain
63/76 (KJ001422) Entoloma phaeocyathus, Spain
99/100 (KJ001419) Entoloma phaeocyathus, Spain
(JX454829) Entoloma aff. minutum, Spain
93/82 (EU784218) Entoloma cuspidiferum, UK
(AF062628) Tricholoma vaccinum, Germany
(AF357060) Lyophyllum decastes, Switzerland
98/100 (HM572546) Lyophyllum decastes, Sweden
outgroup
10

Figure 1. Maximum parsimony tree inferred from internal transcribed spacer nuclear ribosomal DNA sequences showing the position of *Entoloma shandongense*. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Values to the left of / are Maximum Likelihood bootstrap (BS) support, and those to the right indicate the MP bootstrap support (in bold) of that clade. BS values ≥ 50% are shown. *Entoloma shandongense* is placed in bold font to highlight its phylogenetic position. Blue text in the tree represents blue coloured species.

Entoloma shandongense T. Bau & J.R. Wang
(Figures 2–6)

Basidiomata small, omphalinoid. *Pileus* 13–22 mm diameter, convex with a slightly central depression when young, becoming upturned with maturity; surface entirely blue with somewhat purple tint, no colour change with KOH and NH_4OH, non-hygrophanous, smooth to minutely pubescent all over, non-striate; context 1 mm thick, white, no colour change on exposure to air. *Lamellae* short decurrent to decurrent when old, close, creamy-white, becoming yellowish in KOH, 1 mm broad, with lamellulae of 2–3 lengths; edge entire, concolorous with the sides. *Stipe* 2–5 cm long × 3 mm wide, central, equal, fleshy, slightly curved; surface white, smooth; context white, no colour change with KOH, $FeSO_4$ and NH_4OH. *Odour* none. *Taste* mild.

Basidiospores (7.2–)7.5–8.4–9(–10) × 5.4–6.0–6.3(–6.8) µm, Q = 1.14–1.39–1.83, angular with 5–8 angles in profile, hyaline, thick-walled, IKI-, evenly cyanophilic smooth, occasionally with one guttule, with a distinct apiculus. *Basidia* (26–)29–32(–36) × (8–)9–10(–11) µm, clavate to sub-clavate, hyaline, thin-walled, oil drop present when viewed with KOH, 4-spored. *Lamella edge* sterile. and *Cheilocystidia* absent. *Lamellar trama* regular, made up of cylindrical, narrow, parallel elements, 30–70 × 8–12 µm. *Pileipellis* a trichoderm, consisting of thin-walled, erect, often branched hyphae, 5–9 µm diameter, end cells cylindrical, pigment intracellular, plasmatic, dark blue with somewhat purple tinge, sometimes forming clots, which dissolve in 5% KOH. *Pileus trama* composed of cylindrical, hyaline, thin-walled hyphae. *Stipitipellis* composed of filamentous, septate, often branched, thin-walled hyphae, 3–6 µm diameter; *caulocystidia* absent. *Stipe trama* regular, composed of cylindrical hyphae up to 5–7 µm diameter. *Clamp-connections* absent in all tissues.

Habit and Habitat: on soil, under *Ficus religiosa* L. (Moraceae) tree.

Distribution: Previously there was single report on the occurrence of *E. shandongense* from China, Shandong Province, Dezhou City, Botanical Garden of Dezhou, where it was found to grow on soil in *Poa pratensis* L. grassland. The occurrence of this species in India, West Bengal, Kolkata, Golpark Lake, expands its geographical distribution from China to India. As compared to the mentioned ecosystem of the Chinese collection, the Indian specimen was found to grow in a different ecosystem, covered by mostly deciduous trees like *Polyalthia longifolia* (Sonn.) Hook.f. & Thomson (Annonaceae), *Lagerstroemia speciosa* Pers. (Lythraceae) and *Ficus religiosa*.

Material examined: INDIA: West Bengal, Kolkata, Golpark Lake, 22°30′50.4360″ N, 088°21′52.3008″ E, 10 m, 2 August 2014, Ishika Bera, CUH AM109.

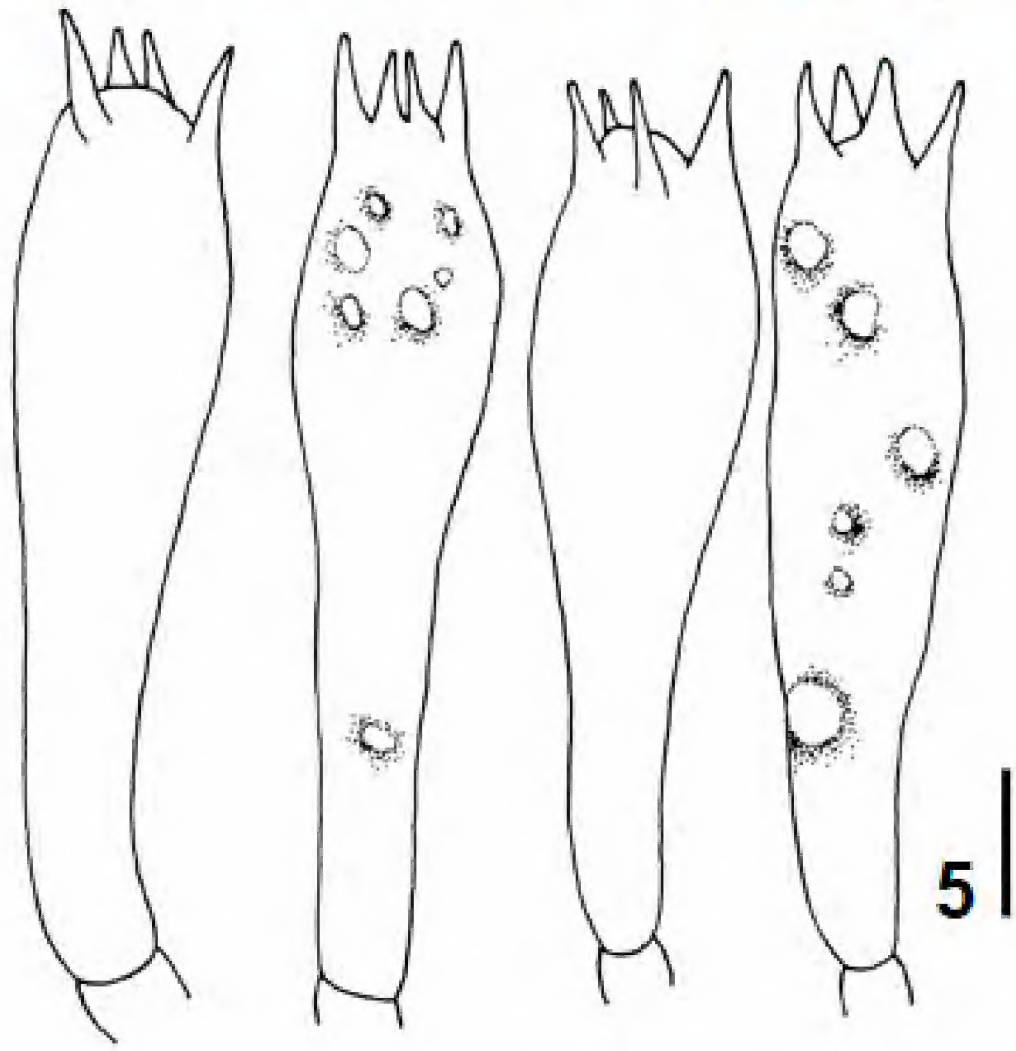
Morphologically the Indian collection matches the type specimen of *E. shandongense*, recently described from Shandong Province, China (Wang and Bau 2013). The distinguishing features of *E. shandongense* include a slightly centrally depressed, overall blue with a somewhat purple tint, non-striate, convex pileus; short decurrent to decurrent, creamy-white lamellae that turn yellowish in KOH, with lamellulae of 2–3 lengths; 5–8 angled in profile view, thick-walled basidiospores (7–10 × 5–7 µm); absence of any cystidia; a trichodermeal pileipellis made up of thin-walled, hyphae 5–9 µm diameter and absence of clamp-connections.

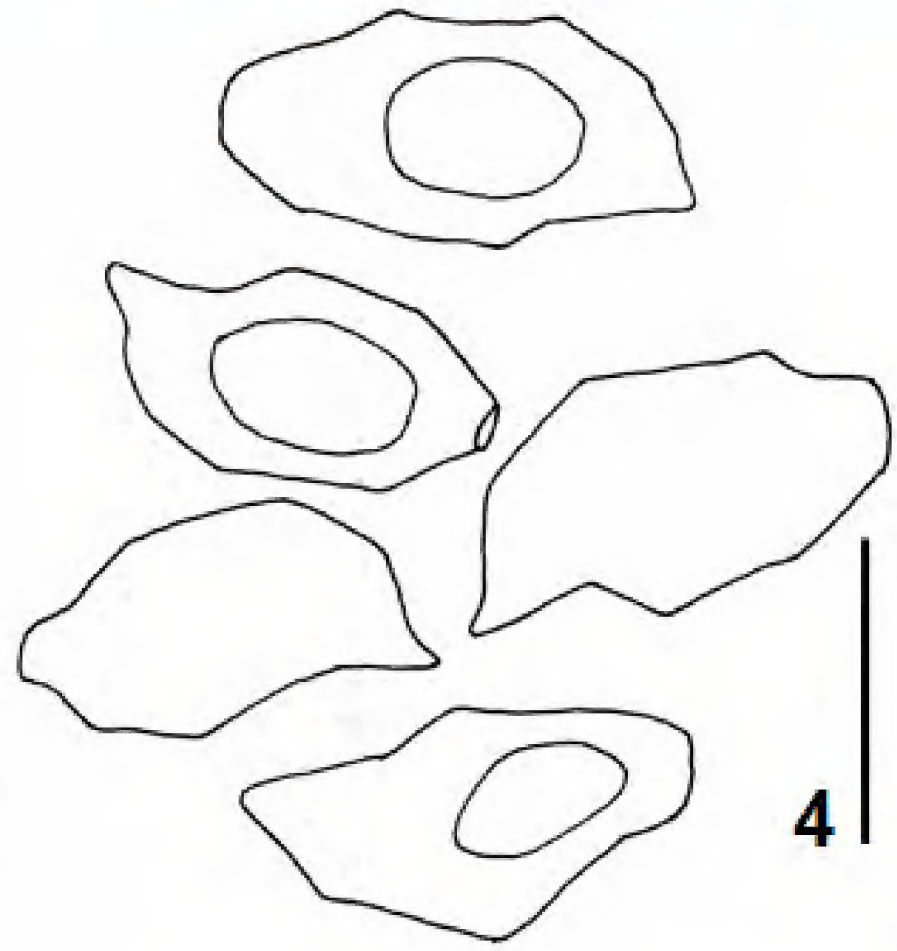
The MP analysis resulted in five equally parsimonious trees with a tree length of 368 steps, CI=0.579288, RI=0.730849, and composite index=0.472669 (0.423372). Figure 1 shows one of the most parsimonious trees. The ML analysis (-lnL = 2394.8031) resulted same topology and relationships for the species groups as like MP. In both analysis (MP and ML), the newly generated sequence of our collected specimen (KP241852) cluster with the type specimen sequence of *E. shandongense* with full support values (Figure 1; MLBS = 100%, MPBS = 100%). Concordant with the micro-morphology, the phylogenetic analysis easily differentiate *E. shandongense* from similar blue coloured *E. atrum* (Figure 1). Unfortunately, there are misidentification problems in some Genbank sequences (see *E. undatum* and *E. byssisedum* in Figure 1).

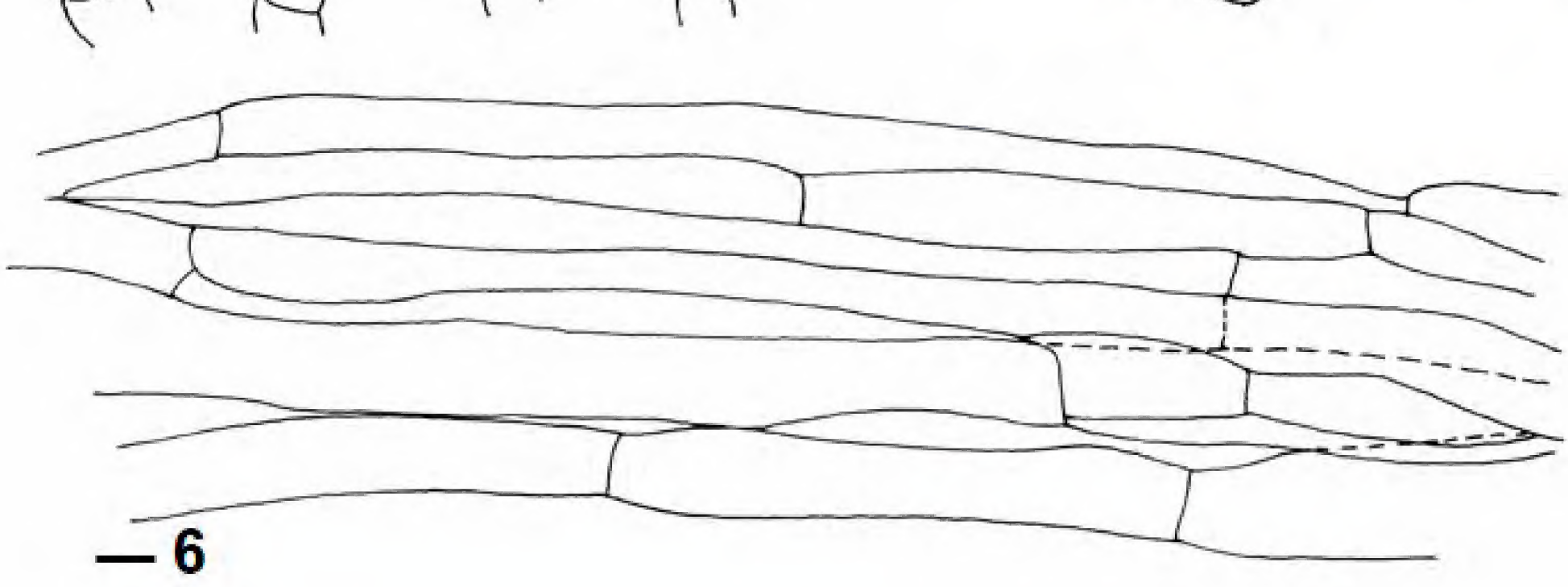
With regard to the blue colouration of the pileus, morphologically *E. shandongense* resembles some of the previously reported taxa from India and its surrounding countries (Manimohan et al. 1995, 2006; Li et al. 2009; Katumoto 2010; He et al. 2012; Pradeep et al. 2012; Wang and Bau 2013). However, there are morphological differences that permit its identification. An artificial key to distinguish *E. shandongense* from its related species is provided.

An artificial key to blue coloured *Entoloma* species reported from India and its surrounding countries

1a	Basidiomata pleurotoid ..... *E. nubilum*
1b	Basidiomata differently shaped 2
2a	Basidiomata omphalinoid; cheilocystidia absent 3
2b	Basidiomata not omphalinoid; cheilocystidia present or absent 4
3a	Basidiospores 7.2–10 × 5.4–6.8 µm .... *E. shandongense*
3b	Basidiospores larger, 10.3–13 × 7.5–8 µm .. *E. atrum*
4a	Basidiospore heterodiametrical to heterodiametrical-ovate shaped 5
4b	Basidiospore differently shaped 8
5a	Cheilocystidia present 6
5b	Cheilocystidia absent 7
6a	Cheilocystidia versiform, mostly clavate or clavate with nodulose apex; caulocystidia absent;







Figures 2–6. *Entoloma shandongense*. **2** and **3**: Habit with mature basidiomata. **4**: Basidiospores. **5**: Basidia. **6**: Pileipellis hyphae. Scale bars: **2**–**3** = 1 cm; **4**–**6** = 5 µm.

basidiospores 11–14 × 6.5–9 µm; clamp-connection present ..... *E. indoviolaceum*

6b Cheilocystidia fusoid; caulocystidia narrowly clavate to fusiform; basidiospores 8–10.5 × 6.5–8 µm; clamp-connection absent ... *E. azureosquamulosum*

7a Pileus and stipe surface appressed squamulose throughout; lamellae adnate; pileipellis a trichoderm ..... *E. suaveolens*

7b Pileus surface velutinous, which becomes fissile toward margin with age; stipe surface with fine longitudinal striations, finely pruinose; lamellae free; pileipellis pluristratous hymeniform ..... *E. griseolazulinum*

8a Cheilocystidia present 9

8b Cheilocystidia absent 12

9a Stipe excessively long, up to 15 cm; cheilocystidia fusoid, with long tapering and apically rounded neck ..... *E. hochstetteri*

9b Stipe not excessively long, upto 8 cm; cheilocystidia almost clavate 10

10a Pseudocystidia present on lamellae edges and sides, sinuous, with slightly constricted-lobed to rounded apex ..... *E. virescens*

10b Pseudocystidia absent 11

11a Pleurocystidia present, clavate; caulocystidia cylindrical shaped; pileipellis a subpalisade; taste bitter ..... *E. altissimum*

11b Pleurocystidia and caulocystidia absent; pileipellis a cutis; taste peppery ..... *E. subaltissimum*

12a Pleurocystidia present; pileipellis a cutis type ..... *E. dinghuense*

12b Pleurocystidia absent; pileipellis not a cutis type 13

13a Pileipellis hymenidermal; basidiospores 9–11 × 6.5–8 µm; clamp-connection absent ..... *E. rugosopruinatum*

13b Pileipellis ixocutis; basidiospores 6–9 × 6–8 µm, subisodiametrical; clamp-connection present ..... *E. nitidum*

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Authors' contribution statement: KA identified the species, wrote the text, SP performed the microscopic work, identified the species, AKD identified the species, drawn the microscopic plate, made the phylogenetic analysis, wrote the text, and IB collected the specimen, prepared macroscopic data.

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